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# Synthesis and characteristics of a novel artificial hapten using the copper mercaptide of penicillenic acid from penicillin G for immunoassay of heavy metal ions

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In this paper, we describe the synthesis of a novel copper ion hapten using the copper mercaptide of penicillenic acid (CMPA) derived from penicillin. Results from tests with immune rabbits indicate that: (i) A new antigen synthesized with CMPA has good stability and is safe for immunizing animals with no toxic phenomena being found in animal experiments; (ii) the immunogenic antigen (CMPA-BSA) can stimulate the immune system to produce specific antibodies with high titrations, up to 150000; and (iii) antibodies in antisera showed higher affinity to OVA-GSH-CuCl than OVA-GSH, which indicates that the antibodies have specific affinity towards copper ions. These results confirm that the novel hapten and relevant antigen for copper ion have been successfully synthesized, giving progress towards an immunoassay for copper ions in environmental and food samples.

**immunoassay, copper ion, hapten, environment, food sample**

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Heavy metals are a class of persistent toxic contaminants and residues found at various levels in the environment and in agricultural and industrial products [1]. Unlike carbon-based contaminants that can be completely degraded to relatively harmless products, metal ions can be transformed in only a limited number of ways through chemical or biological remediation processes [2]. What is worse, through biomagnifications, heavy metal ions existing in the environment can invade the human body and remain there for a long time, posing a serious threat to human health [3]. Copper is an essential element of some metalloenzymes necessary for the normal course of biochemical processes but, like other heavy metals, it is toxic at high concentration [4,5]. All of these factors pose potential risks for the environment and human health [6] and therefore copper and other heavy metals must be monitored and identified in the environment.

The traditional technologies for detecting heavy metals are instrument-based measurements such as atomic absorption spectrometry [7], inductively coupled plasma spectroscopy (ICP-AES and ICP-MS) [8] and stripping voltammetry [9]. Although these conventional instruments accurately detect the amount of metal in an individual sample, they all require expensive laboratory equipment in a centralized facility and sample turnaround is slow. In addition, instrumental analyses provide no information about the metal oxidation state.

In this case, immunoassays based on the affinity and specificity of an antigen-antibody reaction offer an alternative approach in the field of metal ion measurement. Com-

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pared with the traditional detection methods described above, these assays have significant advantages. Immunoassays are time-saving, low-cost, simple to perform, portable, and are feasible for on-site detection. They can also be both highly sensitive and selective. Immunoassays date back to the late 1950s when Yalow and Berson published their work on the development of a quantitative immunological method that could detect human insulin at picogram (pg) levels in small samples of body fluid [6,10,11]. Since then, immunoassays have been widely applied in biology, medicine, agriculture and environment sciences. In the field of heavy metal ion analyses, immunoassays have had a long development process [12–17]. In the 1980s, Meares *et al.* [18–22] first developed a series of methods for synthesizing complexes of metal-chelate-antibodies for clinical medical radio-imaging analysis. Blake *et al.* [12,13,16] performed systemic research into synthesizing monoclonal antibodies to heavy metals by immunizing BALB/c mice with relevant antigens, antibody-based sensors for metal ions detection and relevant heavy metal immunoassays during the following years. To date, immunoassays based on specific antibodies for lead, indium, mercury, uranium and cadmium have been reported [23–29].

The development processes for immunoassays of metal ions include the following procedures: designing and preparing a hapten of metal ions, synthesizing antigen, immunizing animals, screening the antiserum, optimizing the immunoassay, obtaining polyclonal antibodies from the antiserum, selecting a monoclonal antibody with specific affinity to the metal ion and the ultimate application of the monoclonal antibody in the immunoassay for metal ion. Amongst these procedures, the design of the hapten is the key step in the development of an immunoassay method for detection of metal ions in environmental and food samples. It has been shown that a suitable hapten design determines the features of the synthesized antibodies, which in turn determines the specificity and selectivity of the immunoassay [30]. The traditional design concept for metal ion haptens is through utilizing metal ions covalently conjugated to chelators (such as EDTA and DTPA) but in this approach, the monoclonal antibodies induced by the metal-chelate-protein complex preferably bind to the cage-like metal-chelate structure, rather than certain metal ions [28,31]. Wylie *et al.* [17] proposed a new hapten design method by covalently coupling mercuric ion to the –SH group in glutathione. The hapten was subsequently linked to a carrier protein keyhole limpet hemocyanin (KLH) via amide bond. This experiment showed that antibodies induced by the antigen (KLH-GSH-HgCl) could specifically bind to mercuric ions [17]. Penicillin, an unstable compound, could be derived as a stable mercuric or copper mercaptide of penicillenic acid (MMPA or CMPA) and used for self-determination via catalysis of imidazole [32,33]. The derivative of MMPA or CMPA has a unique oxazolone ring instead of the β-lactamic ring in penicillin (Figure 1).

The structure of the oxazolone ring in MMPA/CMPA results in characteristic ultraviolet absorption at about 325 nm [32,33], which could be useful for identification.

In this work, we synthesized a derivative of CMPA based on penicillin degradation [32,33] and successfully used the derivative as a novel copper ion hapten. As will be shown below, the hapten could be used as an effective bi-

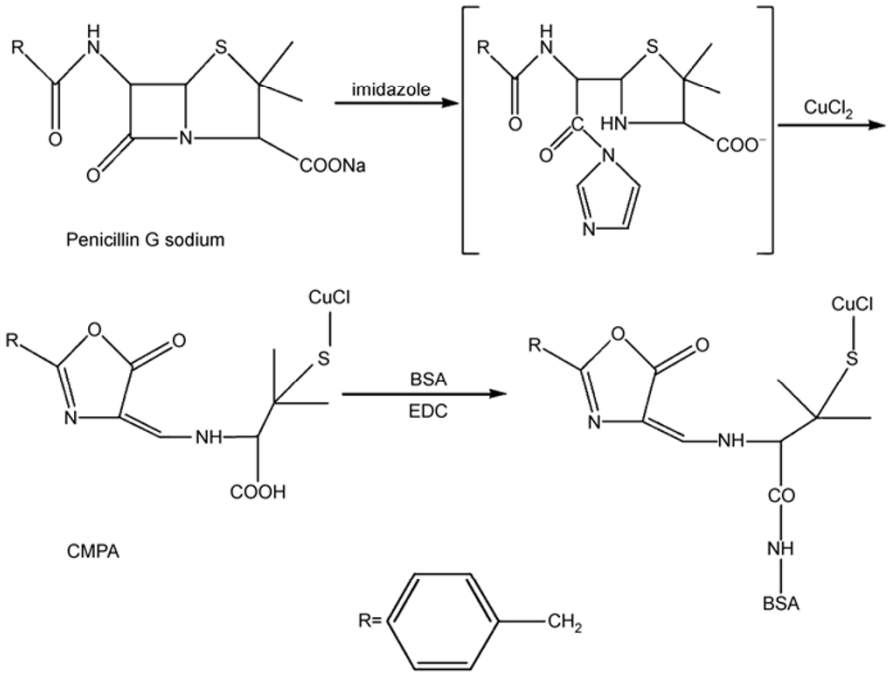


**Figure 1** Synthesis procedures for CMPA and CMPA-BSA from penicillin G.

functional coupling agent to bridge the copper ion and the carrier protein (BSA or OVA), and could stimulate animals to yield high titer antibodies in serum. Below, we describe the relevant synthesis, characteristics, animal immunization and titers of antibodies in serum.

## 1 Materials and methods

### 1.1 Chemicals and reagents

Bovine serum albumin (BSA) was purchased from Lizhu Dongfeng Biotech Co. (Shanghai, China). Reduced L-Glutathione (GSH), imidazole, $CuCl_2$, ascorbic acid and 30% hydrogen peroxide were obtained from Shanghai Chemical Reagents Co. (Shanghai, China). 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC.HCL.) was purchased from BBI Company. Penicillin G (1650 units $mg^{-1}$), Ampicillin, ovalbumin egg (OVA), complete and incomplete Freund's adjuvants were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Tween-20, TMB, goat anti-rabbit IgG conjugated with HRP were purchased from Shanghai Shenhang Chemical Reagents Co. (Shanghai, China). New Zealand Rabbits were purchased from Shanghai SLK Experimental Animal Co. (Shanghai, China). All reagents available were at their highest purity grade.

### 1.2 Apparatus

Infrared (IR) spectra were taken in a Bruker EQUINOX 55 Fourier Transform Infrared Raman Spectroscope (Bruker, Karlsruhe, Germany). ICP-AES measurements were performed on a Thermo Fisher Iris Advantage 1000 inductive coupling plasma emission spectrograph (Thermo Fisher, Waltham, USA). UV and visible spectra were recorded with a Thermo Electron Evolution 300 UV-Vis spectrophotometer. A Shanghai KHB ST-360 Microplate Reader was applied in the ELISA test (Shanghai KHB, Shanghai, China). The pH measurements were made by using a Delta 320 instrument (Mettler Toledo, Zurich, Switzerland).

### 1.3 Buffer and solution

PBS, 50 mmol $L^{-1}$, pH 7.4; carbonate buffer, 50 mmol $L^{-1}$, pH 9.6; PBS washing buffer (PBST): PBS, 50 mmol $L^{-1}$ with 0.05% Tween 20; DMSO, 10% v/v; 4% $NaHCO_3$; substrate solution for horseradish peroxidase: (i) tetramethybenzidin, 10 mg $mL^{-1}$, TMB was diluted in 0.6% DMSO; (ii) pH 5.5 citrate-acetate buffer solution (CPBS: 100 mmol $L^{-1}$ citric acid, 200 mmol $L^{-1}$ $Na_2HPO_4$); (iii) $H_2O_2$, 30%; (iv) 2 mol $L^{-1}$ $H_2SO_4$.

### 1.4 Synthesis of CMPA hapten and antigens

The synthesis procedure for CMPA was described in previous work [32,33]. Brief steps are given below. Step 1: dissolve 40 mg penicillin G and 0.7 g imidazole in 10 mL aqueous solution, which was adjusted with 1 mol $L^{-1}$ HCl to pH 6.80 and gently stirred for 10 min at room temperature. Step 2: add 10 mg $mL^{-1}$ $CuCl_2$ dropwise to the aqueous solution. Step 3: incubate the solution for 1 h at 65°C. Step 4: acidify the solution with 1 mol $L^{-1}$ HCl to obtain a grey precipitate of CMPA as in Figure 2. Step 5: wash the precipitate three times by 1 mol $L^{-1}$ HCl and two times by distilled water to remove unreacte penicillin G and $CuCl_2$.

CMPA was coupled to BSA or OVA via the catalyst EDC/HCl. The amino group in the structure of CMPA was linked to the carrier proteins. To achieve the ideal coupling ratio between CMPA and BSA, a number of factors (e.g., reaction time and reaction molar ratio of BSA and CMPA) were investigated. UV-Vis spectrometry was applied to monitor whether the CMPA was linked to the BSA. ICP-AES was used to determine the coupling ratio of CMPA and BSA. The procedures for synthesis and identification for coating the antigen CMPA-OVA were similar to those of CMPA-BSA.

Using previously described procedures [17], two coating antigens (OVA-GSH and OVA-GSH-CuCl) were synthesized to determine the specific affinity of antiserum to copper ions. ICP-AES was applied to identify the coupling ratio of OVA-GSH-CuCl.

### 1.5 Rabbit and mouse immunization

Four male New Zealand white rabbits were immunized with antigens at a 25∶1 coupling ratio of CMPA to BSA. 1 mg CMPA-BSA was emulsified with Freund's complete adjuvant (1∶1 volume ratio) and injected intradermally at multiple hypodermic sites on the back of each rabbit for the basic immunization. After two weeks, a second immunization was administered with the same emulsified CMPA-BSA. Third and fourth treatments were conducted at biweekly intervals. Each rabbit was bled 7 d after the third immunization, for screening their antisera.

The BALB/c mouse was used for the immunization with the antigen CMPA-BSA above, in accordance with the procedure described in detail by Wylie *et al*. [16,17].

Antisera from these two experiments were isolated via

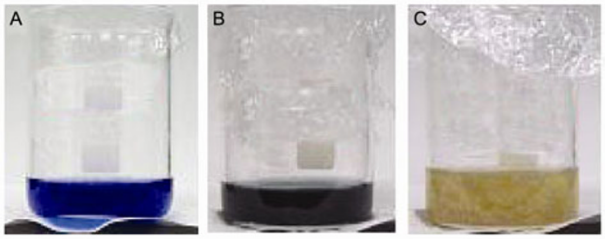


**Figure 2** Mixed solution of the synthesized reaction of CMPA. A, Before water bath at 65°C. B, After the water bath at 65°C. C, After the acidification by 1 mol $L^{-1}$ HCl. The precipitate of the solution is CMPA.

centrifugation with thiomersal added as a preservative and stored at −20°C before further study [34].

### 1.6 Titration test of antisera

Titers of the antiserum from each immune rabbit and mice were screened by indirect noncompetitive ELISA, with negative antiserum as a control according to the following procedure. First, 96-well microtiter plates were coated with 100 μL/well of 5 μg $mL^{-1}$ coating antigen CMPA-OVA in carbonate buffer solution for 3 h in a 37°C water bath. Second, after three washes with PBST, the plates were blocked with 200 μL/well 1% OVA (w/v). After 1 h incubation in a 37°C water bath, the plates were washed as described previously. Third, serial dilutions (1/4000, 1/50000, 1/100000, 1/150000, etc.) of antiserum were conducted in 10 mmol $L^{-1}$ pH 7.4 PBS containing 1% OVA (w/v). 100 μL/well of diluted antiserum was transferred to the plates and, after incubating in a 37°C water bath for 2 h, the plates were washed with PBST. Fourth, 100 μL/well goat anti-rabbit IgG conjugated with horseradish peroxidase (1∶5000 dilution) in 10 mmol $L^{-1}$ pH 7.4 PBS containing 1% OVA (blocking solution) was added and then incubated in the 37°C water bath for 1 h. Fifth, after PBST washing, 100 μL of substrate buffer was added to each well. Sixth, after incubation for 15 min, the reaction was stopped by 2 mol $L^{-1}$ $H_2SO_4$ and the *A* value of each well was read at 450 nm using a Shanghai KHB ST-360 Microplate Reader. The titer dilution of the antibody was considered positive in case where the *A* value was more than 2.1 times that of the negative antiserum.

### 1.7 Analyses of antibodies in antiserum specificity to CMPA and copper

The antibody affinity to CMPA was screened by indirect noncompetitive ELISA. Microtiter plates with 96 wells were coated with 100 μL/well 5 μg $mL^{-1}$ coating antigen CMPA-OVA in CBS. After 3 h incubation in a water bath, the plates were blocked by 1% OVA. Meanwhile, the blank wells were coated with 1% OVA in CBS. Then 100 μL/well antiserum (1/1000 dilution) containing 1% OVA was added. The rest of the steps were similar to those described for the titration test. The absorbance value was read at 450 nm by a Shanghai KHB ST-360 Microplate Reader.

The antibodies' specific affinity to copper ions was further investigated via the coating antigens of OVA-GSH and OVA-GSH-CuCl. Both of these antibodies were diluted to 20 μg $mL^{-1}$ by 50 mmol $L^{-1}$ pH 9.6 carbonate buffer solution (CBS). 100 μL/well OVA-GSH or OVA-GSH-CuCl was added. After 1 h incubation in the 37°C water bath, the plates were blocked by 1% OVA. Then 100 μL/well 1/500 dilution antisera containing 1% OVA was added and incubated for 1 h. The remaining steps were similar to those described above.

## 2 Results and discussion

### 2.1 Characteristics of hapten CMPA

CMPA has a unique oxazolone ring instead of the β-lactamic ring present in penicillin G, resulting in a characteristic UV absorption peak at about 325 nm. The comparison of UV-Vis spectra between CMPA and penicillin G is shown in Figure 3, revealing a characteristic 325 nm absorption peak of CMPA in the precipitate, which confirms the successful synthesis of CMPA. In addition, the analysis of UV-Vis spectra of CMPA conjugated with carrier protein (BSA or OVA) further indicates the existence of the oxazolone ring in CMPA.

IR analysis was also used to confirm the synthesis of CMPA. Figure 4 shows the IR spectra of CMPA and penicillin G. In the spectrum of CMPA, the 1774 $cm^{-1}$ feature band of the β-lactamic ring disappeared, demonstrating that the β-lactamic ring was completely degraded in the process of CMPA synthesis. The existence of –C–C–CO (α, β unsaturated ketone) and –SH (–S–Cu) were shown by characteristic bands at 1665 and 2540 $cm^{-1}$, respectively. Additionally, other nonspecific bands existed in both CMPA and penicillin G sodium, such as at 700, 750 (monosubstitution on aromatic ring), 2927 ($–CH_2$), 1396 and 1400 $cm^{-1}$ [$–C(CH_3)_2$]. Clearly, these results further confirmed the successful synthesis of the hapten CMPA.

Obviously, from the experimental results shown in Figures 3–5, we have successfully synthesized CMPA from penicillin G.

### 2.2 Characterization of CMPA-BSA and CMPA-OVA

For the preparation of animal immune experiments and indirect noncompetitive ELISA, the immune antigen CMPA-BSA and the coating antigen CMPA-OVA were synthesized

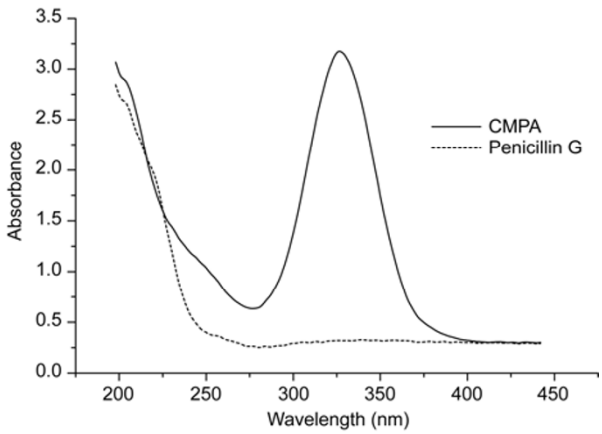


**Figure 3** UV-Vis spectra of penicillin G and hapten CMPA.

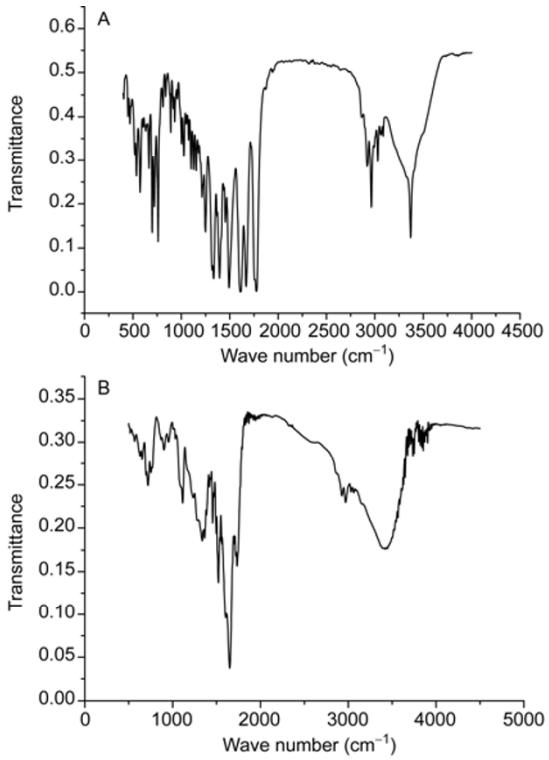


**Figure 4** Infrared spectra of penicillin G (A) and CMPA (B).

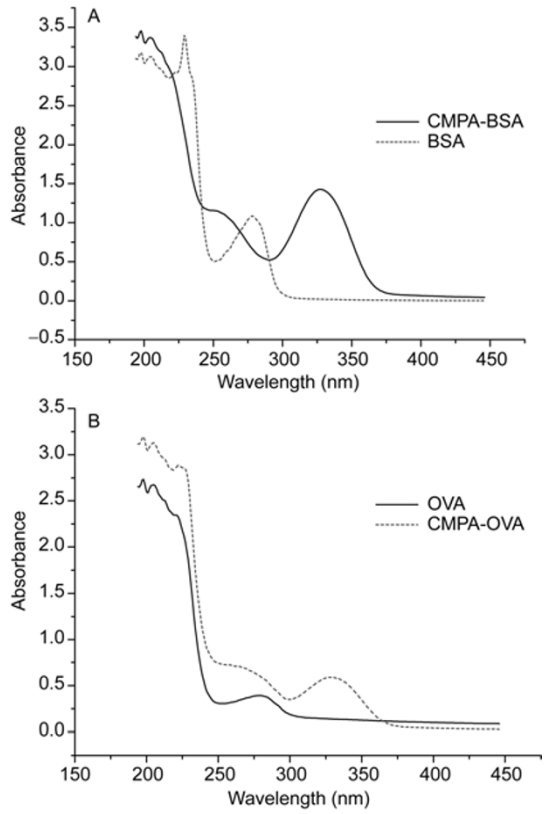


**Figure 5** UV-Vis spectra of CMPA-BSA (A) and CMPA-OVA (B). 0.10 mg mL$^{-1}$ CMPA-BSA and CMPA-OVA solution, or BSA and OVA solution were prepared, then scanned over 190–450 nm wavelengths by a Thermo Electron Evolution 300 UV-Vis spectrophotometer.

and UV-Vis spectrometry was used to identify the conjugation between the hapten CMPA and BSA or OVA. As shown in Figure 5, there is a maximum absorption peak at about 325 nm in the ultraviolet spectra of CMPA-BSA and CMPA-OVA, which implies the successful conjugation between the CMPA and BSA (OVA).

Additionally, because Figure 1 shows the existence of –S–CuCl in CMPA, ICP-AES could be applied to monitor the synthesis of CMPA and the successful linkage between CMPA and BSA or OVA. From the information shown in Tables 1 and 2, we concluded that even after the antigen (CMPA-BSA and CMPA-OVA) purification process by dialysis treatment in PBS, the copper ions are still present in the antigens, indicating the stability of the CMPA. On the other hand, an obvious phenomenon was found during the preparation of the antigen CMPA-BSA: If the reaction molar ratio (CMPA and BSA, *M*) was set at 10∶1, the coupling ratio was constant at 6∶1 after 6 h reaction and further reaction time had no significant influence on the ratio. However, if *M* was set at 100∶1, the coupling ratios would undergo a change from 7∶1 to 24∶1 when the reactive time was increased from 2 to 14 h, respectively. These results clearly demonstrated that the reaction conditions have a strong influence on the coupling ratio of CMPA and BSA. A similar result was found for the synthesis of the coating antigen CMPA-OVA shown in Table 2.

## 2.3 Titers of antisera

As shown in Figure 6, the titer dilution of antibodies in the

**Table 1** Influence of synthesis conditions on binding ratio of CMPA∶BSA[a)]

| Reaction time (h) | Coupling ratio | |
|---|---|---|
| | (*M*=10∶1)[b)] | (*M*=100∶1)[b)] |
| 2 | 4∶1 | 7∶1 |
| 6 | 6∶1 | 11∶1 |
| 12 | 6∶1 | 20∶1 |
| 14 | 6∶1 | 24∶1 |

a) After reaction, unreacted CMPA was removed by dialysis. Then, antigen solution was diluted to a certain concentration. Copper ion concentration of CMPA-BSA was detected by ICP-AES. b) *M* represents the molar ratio between CMPA and BSA before reaction.

**Table 2** Influence of synthesis conditions on binding ratio of CMPA∶OVA[a)]

| Reaction time (h) | Coupling ratio | |
|---|---|---|
| | (*M*=10∶1)[b)] | (*M*=100∶1)[b)] |
| 2 | 4∶1 | 3∶1 |
| 6 | 4∶1 | 5∶1 |
| 12 | 4∶1 | 5∶1 |
| 14 | 4∶1 | 5∶1 |

a) After reaction, unreacted CMPA was removed by dialysis. Then, antigen solution was diluted to a certain concentration. Copper ion concentration of CMPA-OVA was detected by ICP-AES. b) *M* represented the molar ratio between CMPA and OVA before reaction.

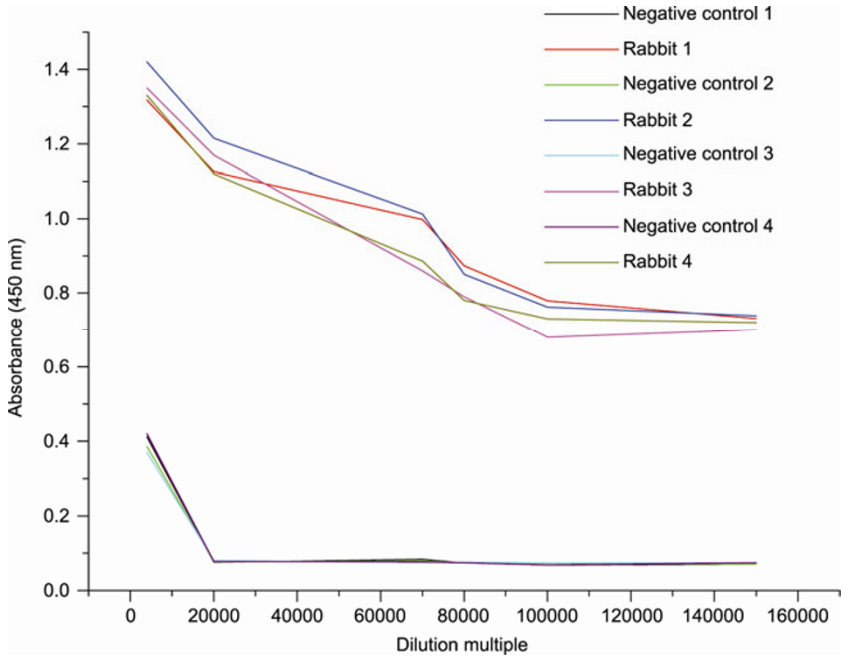


**Figure 6** Serum's titration test of two rabbits after final boosting immunization (binding ratio of CMPA-BSA was 24∶1). We used indirect ELISA here. Before test, immunizing serum and normal serum for each rabbit were diluted 4000, 20000, 70000, 80000, 100000, and 150000 times.

antisera was considered positive when the *A* value at 450 nm was more than 2.1 times that of the negative control antiserum. It was clearly revealed that (i) the titer of antibodies in the antisera was high, even if the antiserum dilution was as high as 150000; (ii) among four immune animals, rabbit 2 showed the highest titers of antiserum, which implies that rabbit 2 antiserum is the optimal candidate for subsequent experiments.

### 2.4 Affinity of the antiserum to CMPA

In this section, the affinity of the antibodies in rabbit antiserum to CMPA was detected via indirect noncompetitive ELISA. Figure 7 shows results that demonstrate the antiserum's affinity to CMPA. Compared with the titers of the negative control serum and the good coating with 1% OVA, the antibodies had a higher affinity to the coating antigen CMPA-OVA.

Furthermore, the ELISA determination of the mouse antiserum was processed to investigate the affinity of antibodies in antiserum to CMPA. The results listed in Table 3 indicate that (i) antibodies of five mice antisera all displayed a low level of affinity to the coating antigen OVA; (ii) it was observed that the antisera of mouse 4 and 5 showed favorable titers with higher affinity towards the antigen CMPA-OVA.

These results demonstrate that our novel synthesized hapten CMPA had immunogenicity that stimulated the animals' immune systems to produce the high affinity antibody.

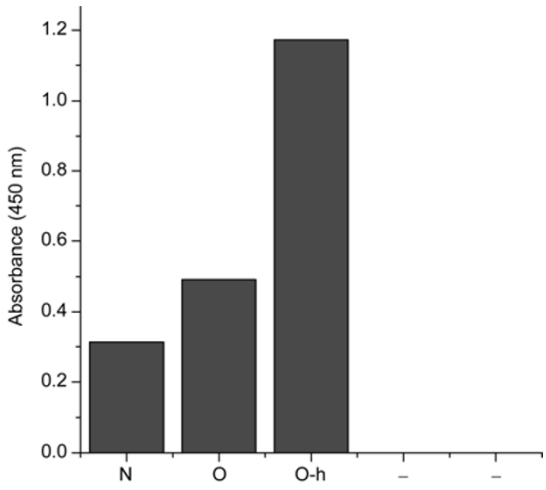


**Figure 7** Affinity of antibody in antiserum to CMPA. N, Normal serum for negative control; O, immunized serum, coating with antigen was 1% OVA; O-h, immunized serum, coating antigen was OVA-CMPA. Before the ELISA analyses, antisera were diluted 1000-fold. The blank wells (O) were coated with OVA. Wells (O-h) coated with antigen CMPA-OVA were utilized to detect specific affinity to CMPA.

### 2.5 Specific affinity of serum antibody towards copper

After completing the experiments above, we further investigated the specific affinity of antibodies in antiserum against copper ions. We synthesized two coating antigens, OVA-GSH and OVA-GSH-CuCl, through related research work [17,35]. In the structure of OVA-GSH-CuCl, the coupling ratio was 1∶5, as detected by ICP-AES. As shown in

**Table 3** The antibody titer in mouse antiserum by ELISA after the third boosting immunization with CMPA-BSA

| Mouse | Neutralized with | Antibody titer | |
|---|---|---|---|
| | | CMPA-BSA[a)] | BSA[a)] |
| No. 1 | BSA | <1000 | <1000 |
| No. 2 | BSA | <1000 | <1000 |
| No. 3 | BSA | <1000 | <1000 |
| No. 4 | BSA | 1000 | <1000 |
| No. 5 | BSA | 2000 | <1000 |
| Normal | BSA | <1000 | <1000 |

a) The antigen (10 μg $mL^{-1}$) was coated on the ELISA plate.

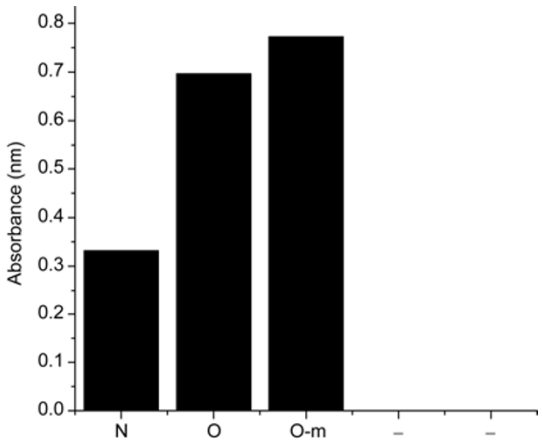


**Figure 8** Affinity of antibody in the antiserum of rabbit 2 for copper ions. N, Normal serum for negative control; O, immunized serum, with coating antigen OVA-GSH; O-m, immunized serum, with coating antigen OVA-GSH-Cu.

Figure 8, the affinity of antibodies in serum of rabbit 2 to OVA-GSH-CuCl showed a favorable titer with an $A_{450}$ value of 0.771, which is higher than that of the negative control serum ($A_{450}$, 0.332) and wells coated with antigen OVA-GSH ($A_{450}$, 0.692). The different affinities of the antiserum towards OVA-GSH-CuCl and OVA-GSH indicates that the antibodies specific to the copper ion existed in the antiserum of the rabbit. The detailed results of the four rabbits are shown in Table 5, indicating that the antibodies (rabbit) induced by the antigen CMPA-BSA had a specific affinity against copper ions.

The detailed results regarding whether antibodies of the immunized mouse antiserum have specific affinity against copper ions are shown in Table 4. The antiserum was neutralized with BSA before applying the ELISA. Subsequently, the titers of the antibody to the CMPA-BSA, BSA, Cu-GSH-OVA and GSH-OVA were determined. It can be observed in Table 4 that the five immunized mice 4 and 5 produced high affinity antibodies to the CMPA hapten. However, there was no obvious difference observed between the antibodies' affinities against OVA-GSH-CuCl and OVA-GSH in the antisera of mice.

**Table 4** ELISA test on antibody titer in mouse antiserum after 3rd boosting immunization with CMPA-BSA

| Mouse | Neutralized with | Antibody | | Titer[a)] | |
|---|---|---|---|---|---|
| | | CMPA-BSA[b)] | BSA[b)] | Cu-GSH-OVA[b)] | GSH-OVA[b)] |
| No. 1 | BSA | <1000 | <1000 | <1000 | <1000 |
| No. 2 | BSA | <1000 | <1000 | <1000 | <1000 |
| No. 3 | BSA | <1000 | <1000 | <1000 | <1000 |
| No. 4 | BSA | 1000 | <1000 | 1000 | 1000 |
| No. 5 | BSA | 2000 | <1000 | 1000 | 1000 |
| Normal | BSA | <1000 | <1000 | <1000 | <1000 |

a) The first dilution of serum sample with PBS was 1000. b) The antigen (CMPA-BSA) was coated on the ELISA plate.

**Table 5** Comparisons of the *A* values between OVA-GSH and OVA-GSH-CuCl coating antigens

| Rabbit | OVA-GSH | OVA-GSH-CuCl | Relative difference[a)] |
|---|---|---|---|
| No. 1 | 0.657±0.035 | 0.689±0.042 | 4.8 |
| No. 2 | 0.692±0.032 | 0.771±0.034 | 11.4 |
| No. 3 | 0.626±0.028 | 0.639±0.026 | 2.07 |
| No. 4 | 0.682±0.039 | 0.724±0.046 | 6.15 |

a) The relative difference was calculated by the formula: [($A_{450}$ of OVA-GSH-CuCl–$A_{450}$ of OVA-GSH)/($A_{450}$ of OVA-GSH)]×100%. Each number represents the average of triplicate determinations.

Both the results of rabbit and mouse immune experiments indicate that there is a weak specific affinity existing between the antibodies in antiserum and copper ions. The weak affinity of antibody to copper ions might be caused by the following. First, in the structure of CMPA, there are three epitopes: the oxazolone ring, the phenyl ring and the –S–CuCl group. Compared with the former two groups with strong immunogenicity, the –S–CuCl group has only a weak impact on the animal immune system. Because of the large size of the oxazolone ring and the phenyl ring, the –S–CuCl epitopes shielded by these two groups and there are few opportunities for recognition by the animal immune system. Thirdly, the connecting arm bridging between the copper ion and carrier protein BSA in the molecular structure of CMPA is –S–C–C–CO– (shown in Figure 1), which is not long enough to give the copper ion of CMPA full exposure to the immune system of animals and this strengthens the space shielding efficiency of the other groups around the

–S–CuCl epitope [17,30].

Two approaches were taken to solve this problem. Initially, through the production of monoclonal antibody, we obtained an antibody with highly specific affinity to copper ions, but it is a time-consuming process and usually requires expensive equipment and reagents [34]. Second, we could add a sample pretreatment step before starting the copper ion immunoassay based on antibodies induced by CMPA. In our lab, we have successfully synthesized two types of metal ion hapten: CMPA for copper ions and a mercuric mercaptide of penicillenic acid (MMPA) for mercury ions [31]. However, the synthesis system used for mercury and copper ion haptens is not suitable for other metal ions, e.g., Pb(II), Cr(III), Co(II), Cr(III), Pb(III), Ni(II). Furthermore, with a simple pretreatment of sample to remove mercury ions, we could utilize the antibody recognition for copper ions with CMPA instead to determine the concentration of copper ions in environmental and food samples. This method may offer a novel strategy for the immunoassay of heavy metal ions in the future.

## 3 Conclusion

In this paper, based on prior advances [32,33,35,36], we successfully synthesized a novel copper ion hapten CMPA from a penicillin derivative. The final results indicate that (i) CMPA is stable both *in vitro* and *in vivo* and is safe for immunized animals, with no toxic phenomena found during immunizations; (ii) the novel synthesized antigen (CMPA-BSA) could stimulate the immune system to produce specific affinity with high titrations up to 150000; (iii) antibodies in the antisera showed a higher affinity against coating antigen OVA-GSH-CuCl than that against OVA-GSH, indicating the existence of the specific antibodies against copper ions in the antisera. In addition, we found that the coupling ratio of CMPA and BSA has a significant influence on the immune response of animals, in accordance with a previously determined principle, namely that a higher coupling ratio usually increases the strength and specificity of the immune response [30,37].

Our results show that the novel synthesized copper antigen (CMPA-BSA) clearly has potential as an immunoassay for detecting copper ions in the environment and food samples. Meanwhile, this novel method for synthesis of a hapten for copper ions opens up a new way forward for the design of haptens for heavy metal ion detection by immunoassay.

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